NIEHS Microarray User's Group Meeting: Factors Affecting RNA Quality Sample Submission Process

September 21, 2001

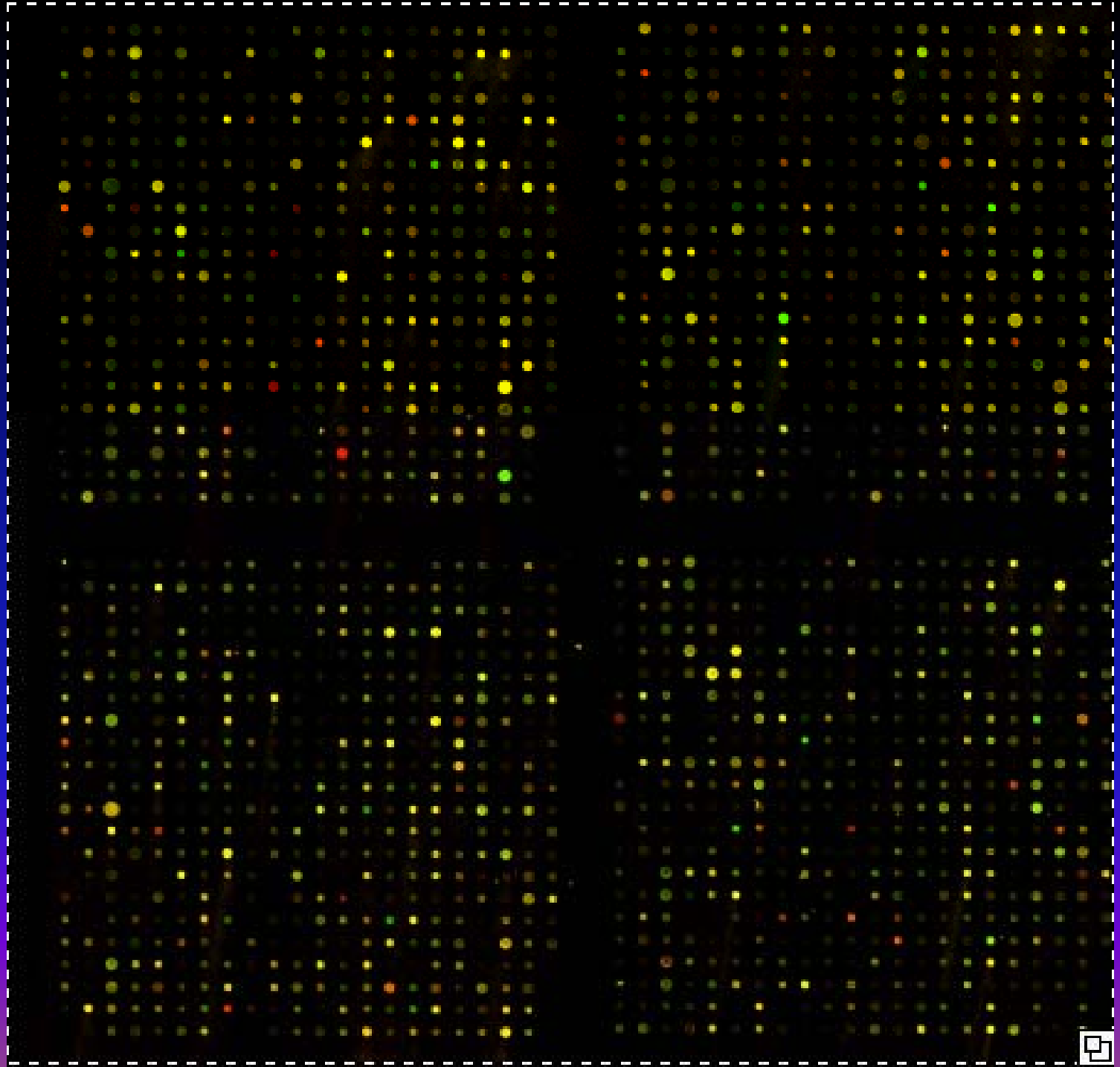
Cindy Afshari, Ph.D.

Outline

- How critical is RNA quality?
- Considerations for isolating high quality RNA
- Assessing RNA quality
- Submitting samples to the microarray lab

How critical is RNA quality?

- RNA quality may be the biggest factor in determining the “picture perfect” hybridization
- RNA quality affects labeling efficiency
- RNA quality affects signal to noise ratio/background contribution



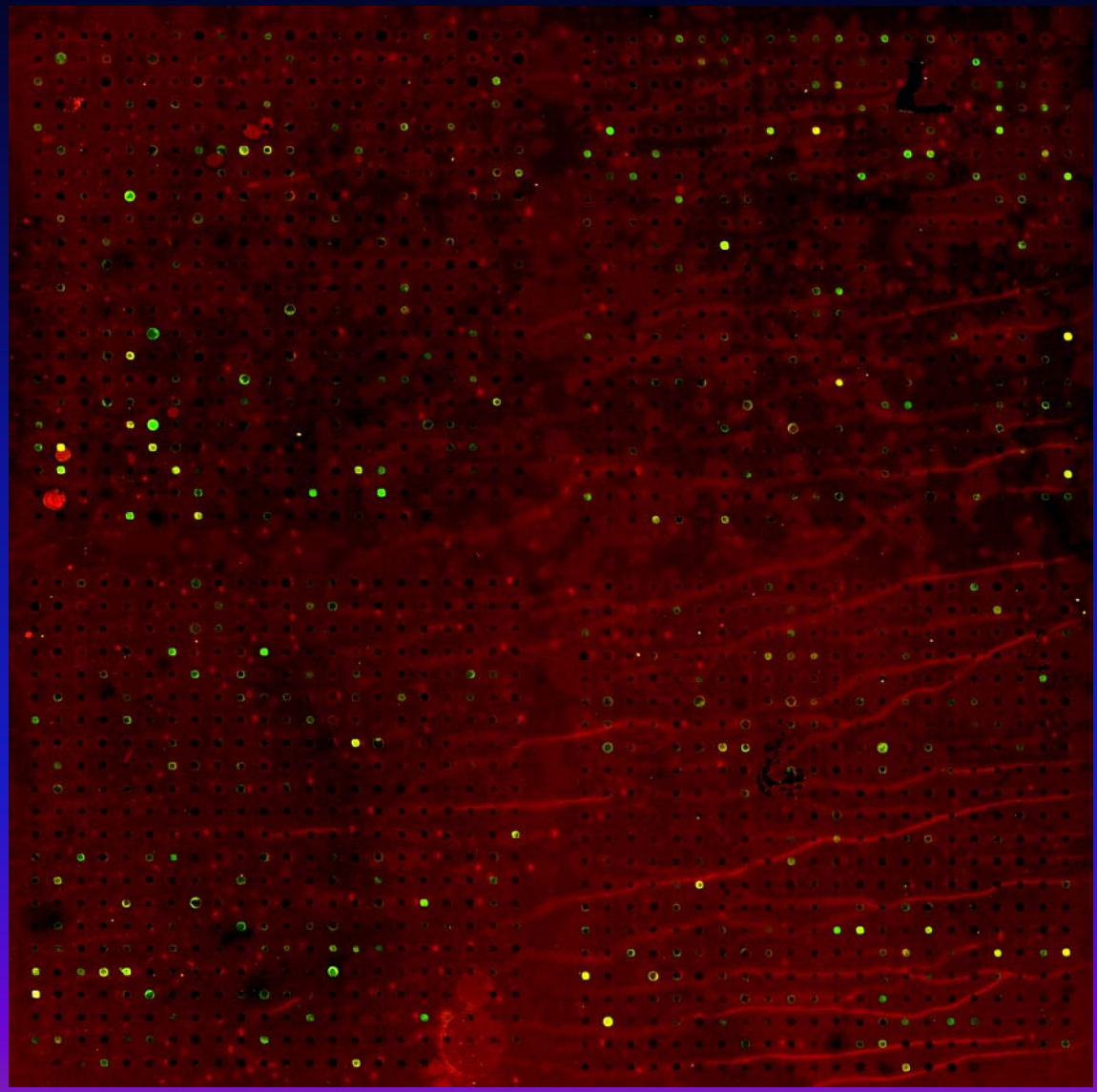
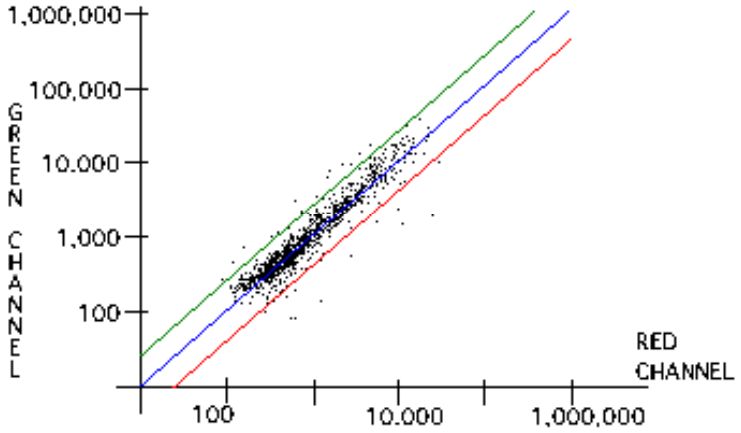


Image Name: p30s13c3.Tif
p30s13c5rotflpmarked



○ Histogram/ ◉ Scatter Plot

Data from:
- ◉ All targets
- ○ Control targets

Calibration by:
- ○ Internal Controls
- ◉ All targets
- ○ Background

Calibration Method:
- ◉ Ratio Distribution
- ○ Log-Normal

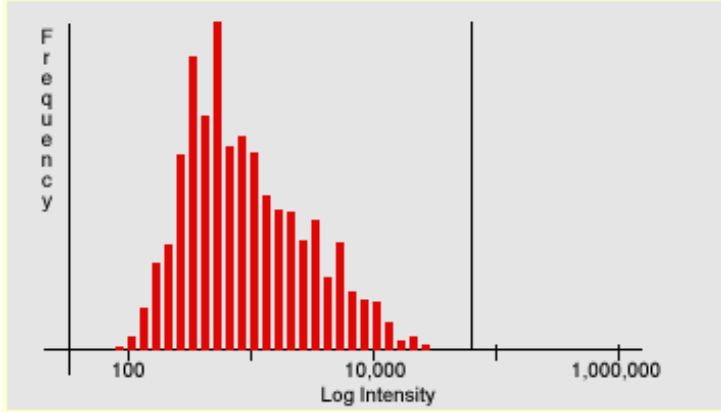
☑ Log Scale ☐ Calibrated Result

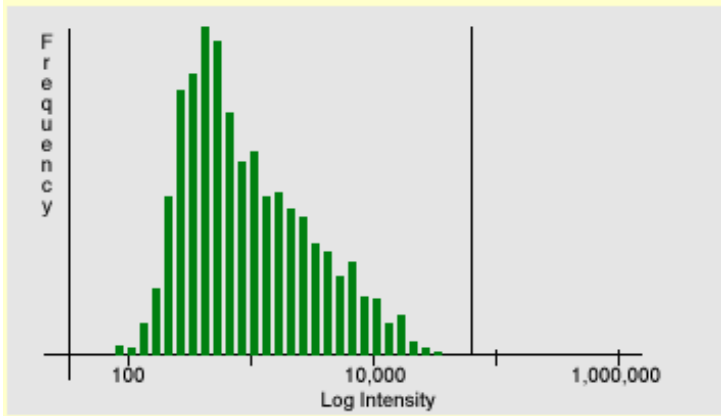
Confidence Level:	99.00	Intensity From:	1
Ratio Lower Limit:	0.38	Intensity To:	65535
Ratio Upper Limit:	2.45	Target Size From:	30

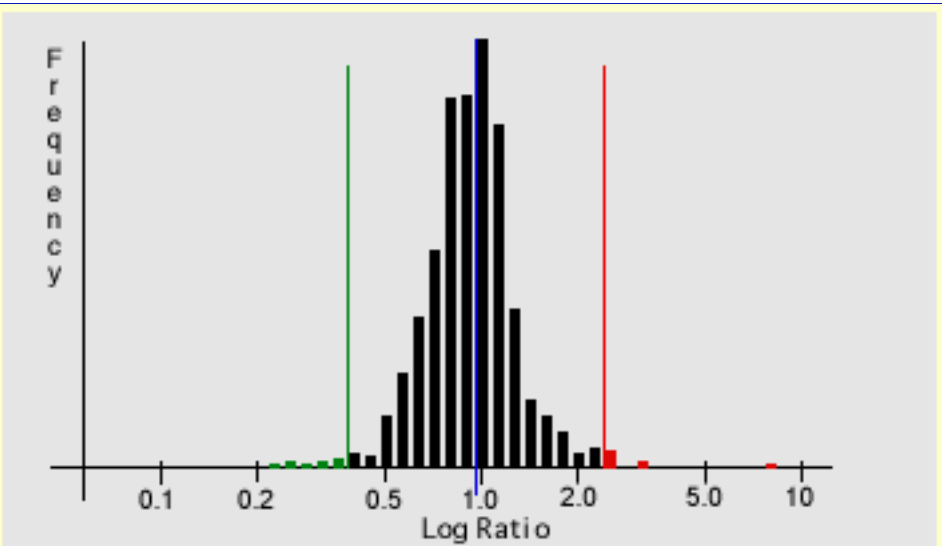
Refine Stats

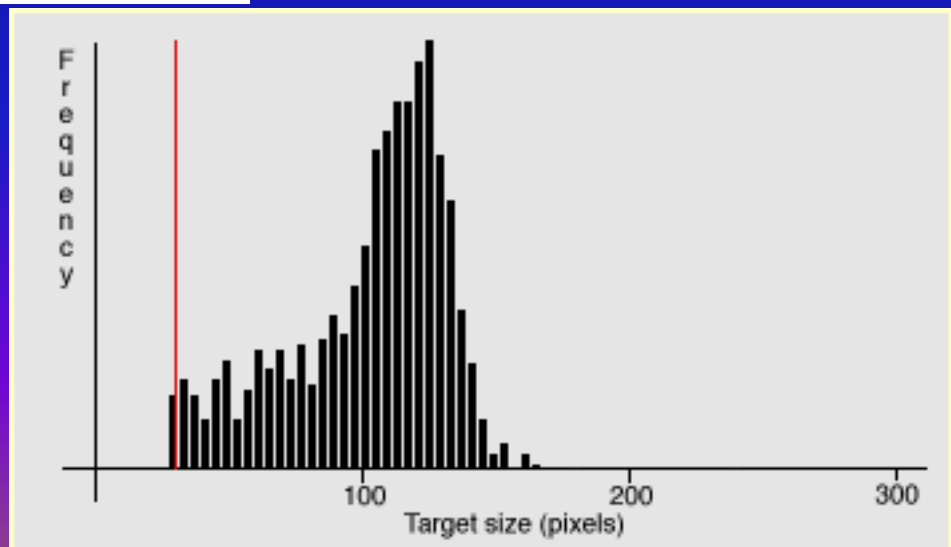
CV = 0.218
M = 0.972

Default Setting | Ratio Outliers | Update dataSheet | Equal Intensities | Exit | Refresh





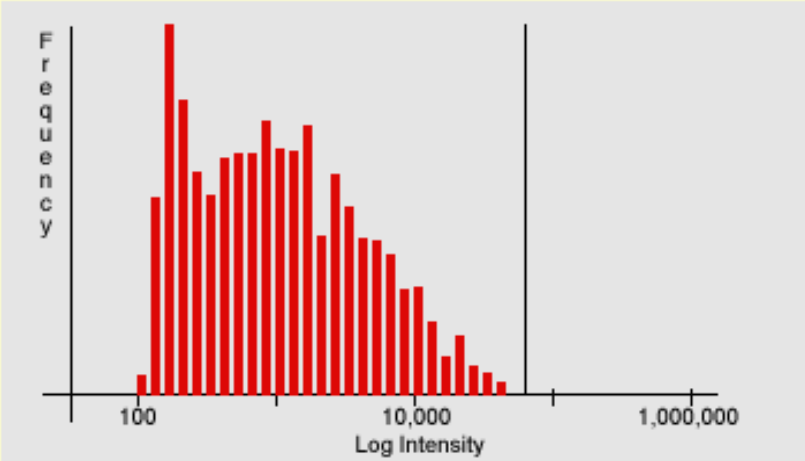




File Edit View Enhance Analyze Math Ext. Window Help

Color Image(enhaced)

Image Name: p21s85_532_flpmrk.IP
p21s85_635_flp.IP



Histogram/ Scatter Plot
Ratio
Red channel
Green channel
Size

Data from:
All targets
Control targets

Calibration by:
Internal Controls
All targets
Background

Calibration Method:
Ratio Distribution
Log-Normal

Log Scale
Calibrated Result

Confidence Level:	99.00	Intensity From:	1
Ratio Lower Limit:	0.36	Intensity To:	65535
Ratio Upper Limit:	2.47	Target Size From:	30

Refine Stats

CV = 0.223
M = 0.949

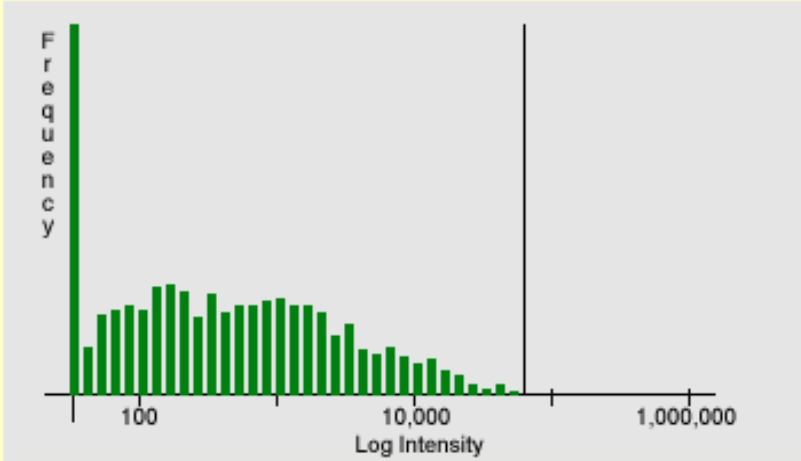
Default Setting
Ratio Outliers
Update dataSheet
Equal Intensities
Exit
Refresh

TargetLocator, 1.3, © 1997, NIH/NHGRI/LCG

File Edit View Enhance Analyze Math Ext. Window Help

Color Image(enhaced)

Image Name: p21s85_532_flpmrk.IP
p21s85_635_flp.IP



Histogram/ Scatter Plot

Ratio
Red channel
Green channel
Size

Data from:
All targets
Control targets

Calibration by:
Internal Controls
All targets
Background

Calibration Method:
Ratio Distribution
Log-Normal

Log Scale
Calibrated Result

Confidence Level:	99.00	Intensity From:	1
Ratio Lower Limit:	0.36	Intensity To:	65535
Ratio Upper Limit:	2.47	Target Size From:	30

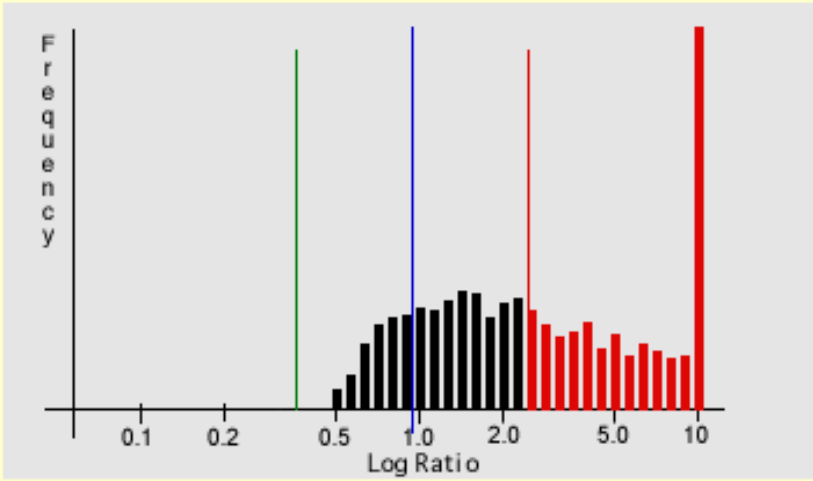
Refine Stats

CV = 0.223
M = 0.949

Default Setting
Ratio Outliers
Update dataSheet
Equal Intensities
Exit
Refresh

Color Image(enhaced)

Image Name: p21s85_532_flpmrk.IP
p21s85_635_flp.IP



Log Scale

Calibrated Result

Confidence Level:	99.00	Intensity From:	1
Ratio Lower Limit:	0.36	Intensity To:	65535
Ratio Upper Limit:	2.47	Target Size From:	30

Default Setting | Ratio Outliers

Update dataSheet | Equal Intensities

Histogram/ Scatter Plot

Ratio
Red channel
Green channel
Size

Data from:
All targets
Control targets

Calibration by:
Internal Controls
All targets
Background

Calibration Method:
Ratio Distribution
Log-Normal

Refine Stats

CV = 0.223
M = 0.949

Exit | Refresh

TargetLocator, 1.3, © 1997, NIH/NHGRI/LCG

Most commonly observed problems with RNA

- Degradation
- Quality/DNA contamination - gel
- Quantitation - concentration
- Low mRNA level/high rRNA
- pH/salt - some DEPC and sodium acetate
- Protein/glycolipid contamination

Important Steps in RNA Isolation

- Tissue handling
- Tissue storage
- Tissue disruption
- RNA isolation
- RNA quantitation
- RNA storage

Important Steps in RNA Isolation

- Tissue handling:
  - Culture or animal handling for harvest/necropsy
  - Freezing of cells/tissue
    - Size of tissue sample
    - Liquid nitrogen
    - RNA Later (Ambion)

Important Steps in RNA Isolation

- Tissue storage:
  - Liquid nitrogen
  - RNA later
- Store until lysis buffer is added

Important Steps in RNA Isolation

- Tissue disruption:
  - Sonicator, polytron, homogenizer
    - (see Julie Foley's presentation)
  - Poor disruption will affect amount and quality of yield

Important Steps in RNA Isolation

- RNA isolation
  - Qiagen RNAeasy
  - Triazol or other methods
- Don’t overload columns or volumes of solutions!!!!!
- Be careful to reduce contamination from salts, ethanol, or organic solvents (phenol).

Important Steps in RNA Isolation

- RNA quantitation
  - Consider a standard, commercial source to calibrate spectrophotometer
- RNA concentration

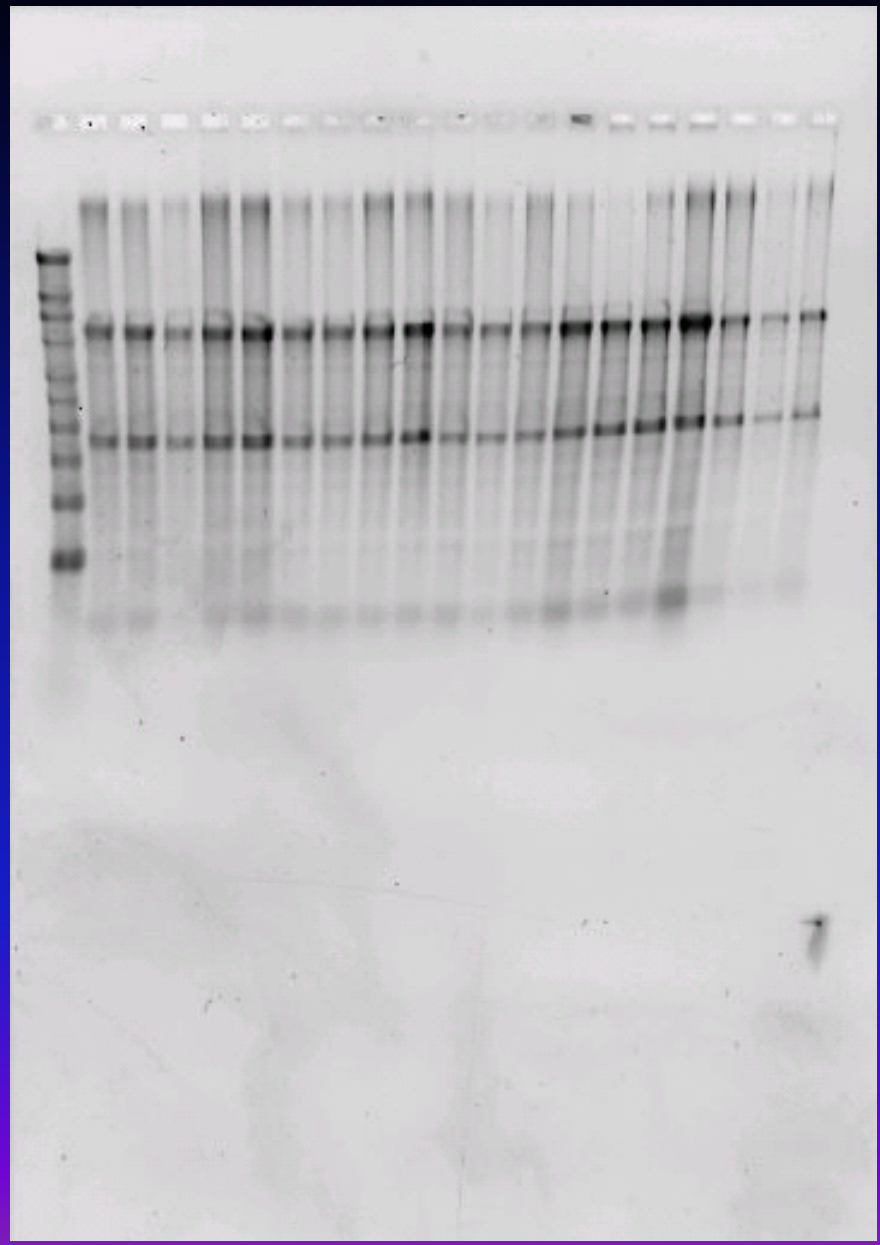
Important Steps in RNA Isolation

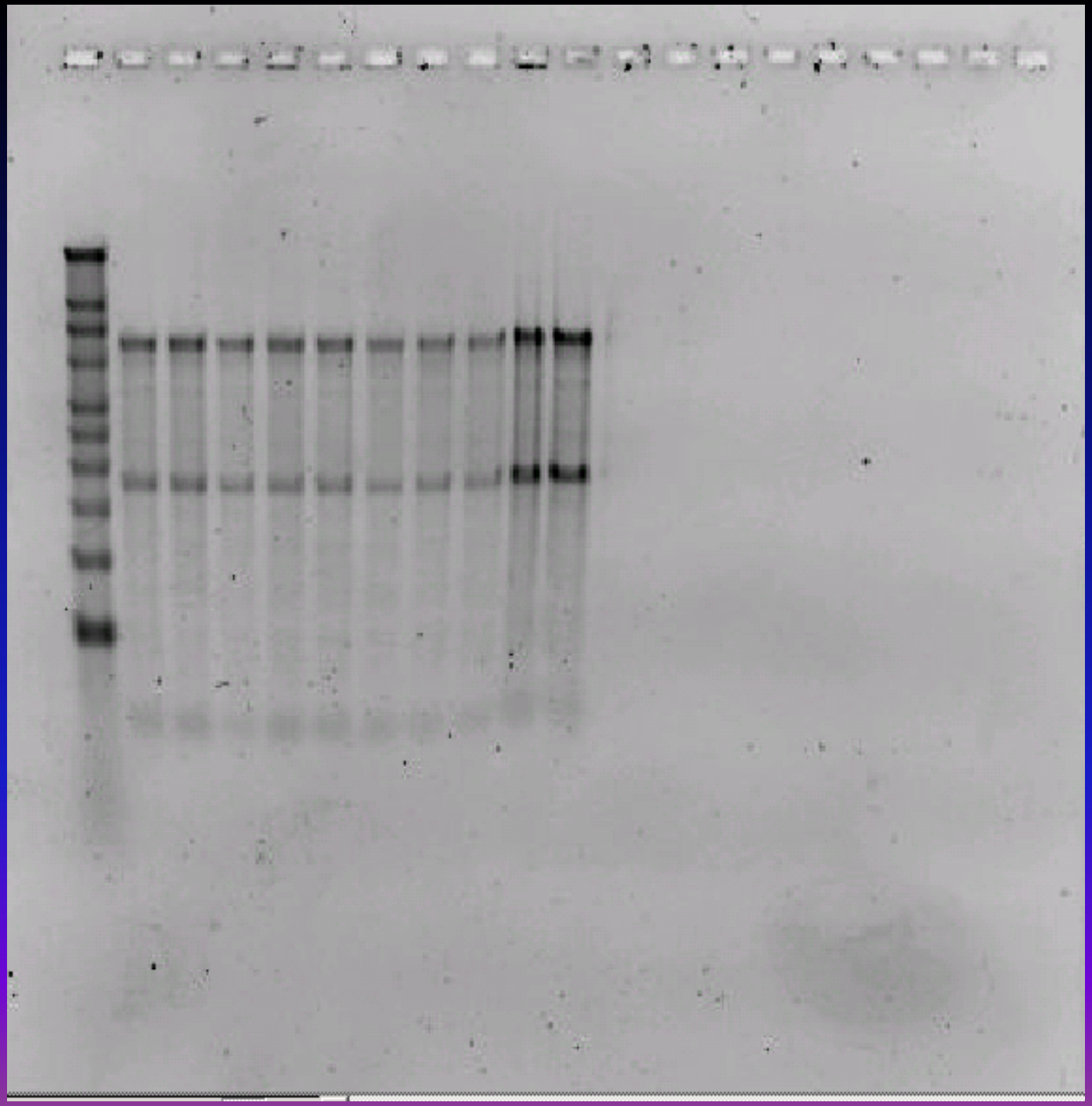
- RNA storage:
  - -80 degrees
  - Move on dry ice
  - Aliquot if large amount
  - Thaw on ice

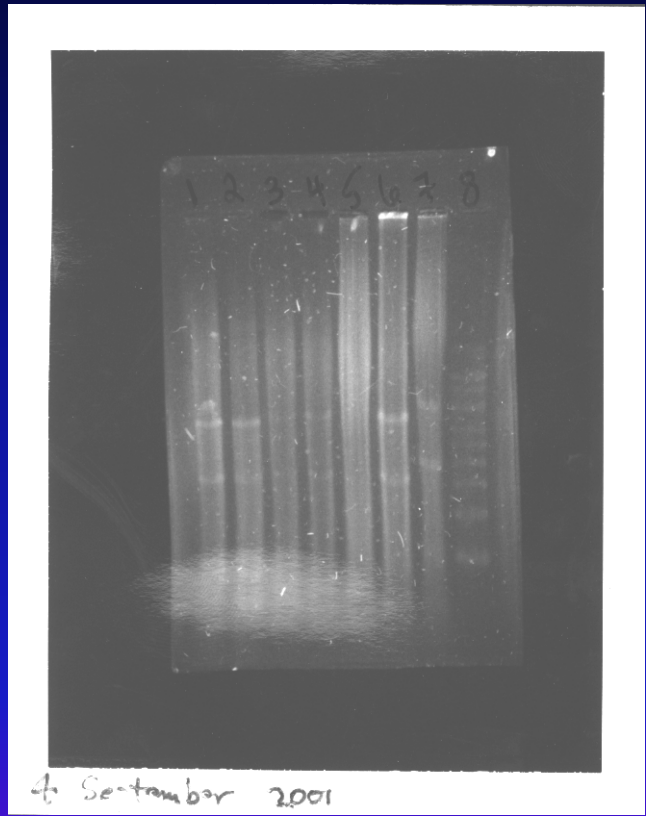
Assessing RNA Quality

- OD 260/280 Ratio: 1.8- 2.0
- Ethidium bromide stained formaldehyde agarose gel
- Agilent Bioanalyzer

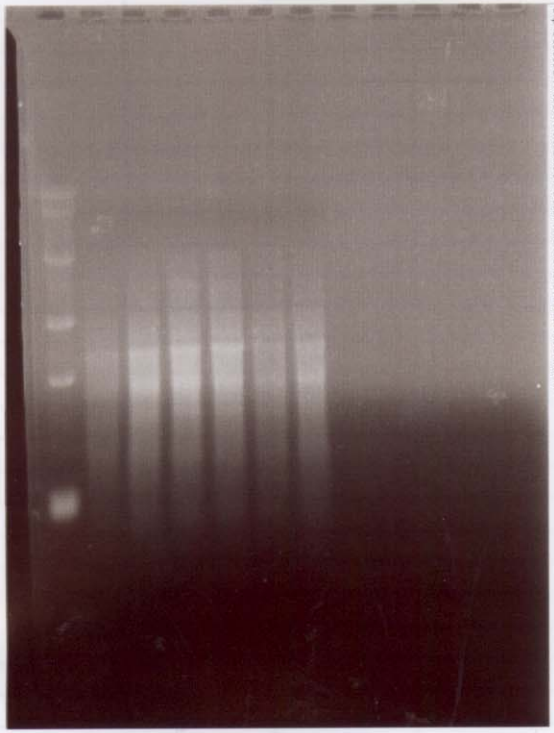
1 2 3 4 5






1 2 3 4 5 6 7 8
4 September 2001


SFDM
V_2O_5
H_2O_2
5/17/91



Lane	
1	RNA ladder
2	BG-1 Control 2hr
3	BG-1 & 2 2hr
4	BG-1 control 6hr
5	BG-1 & 2 6hr
6	BG-1 control 24hr
7	BG-1 & 2 24hr

RNA: Human Hearts 8/22/00

001a 001b 004a 004b 005a 005b 016a 016b

001 = Control 6/6/00
004 = Failure 6/22/00
005 = Failure 6/13/00
016 = Failure 7/11/00

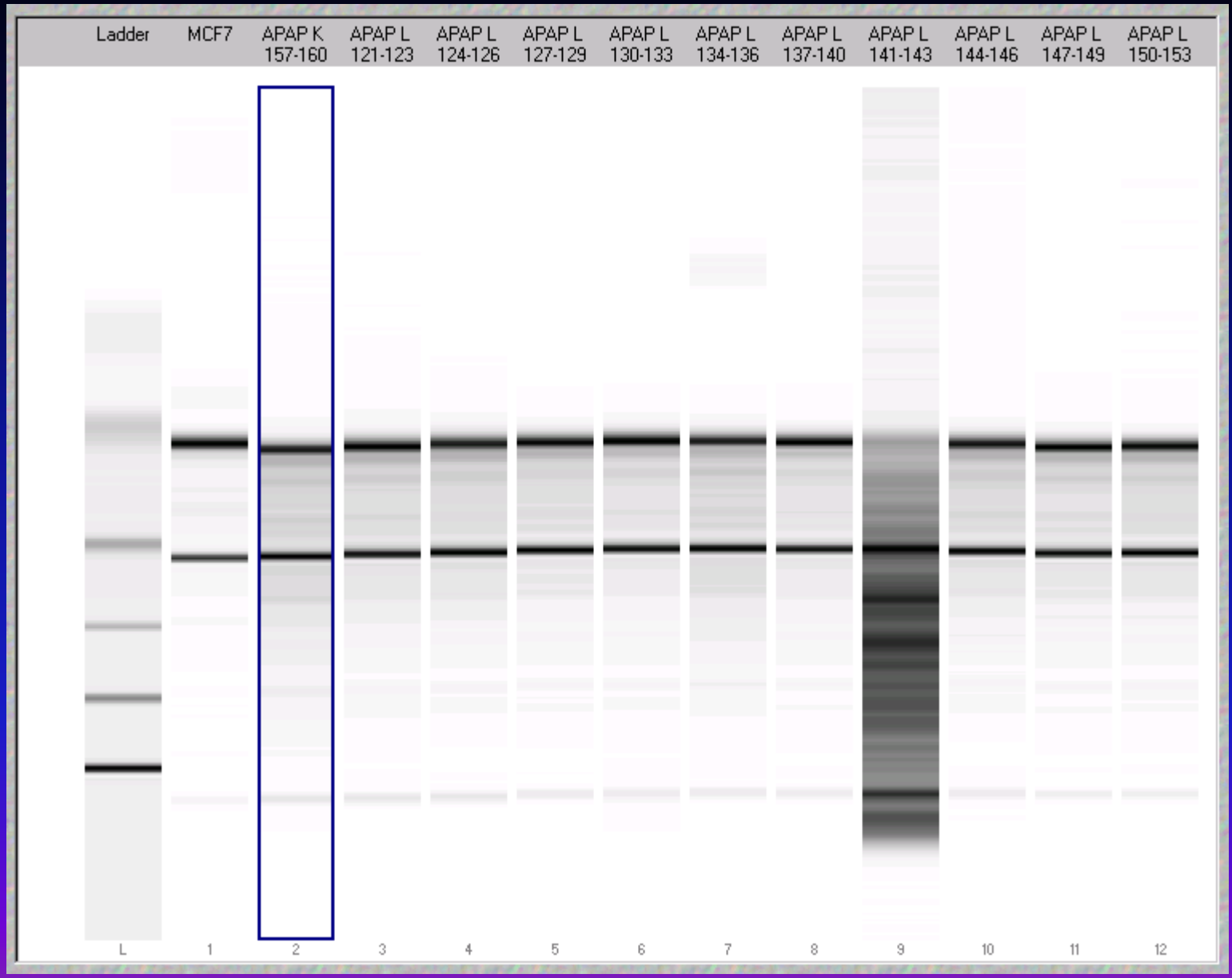
4 hr.

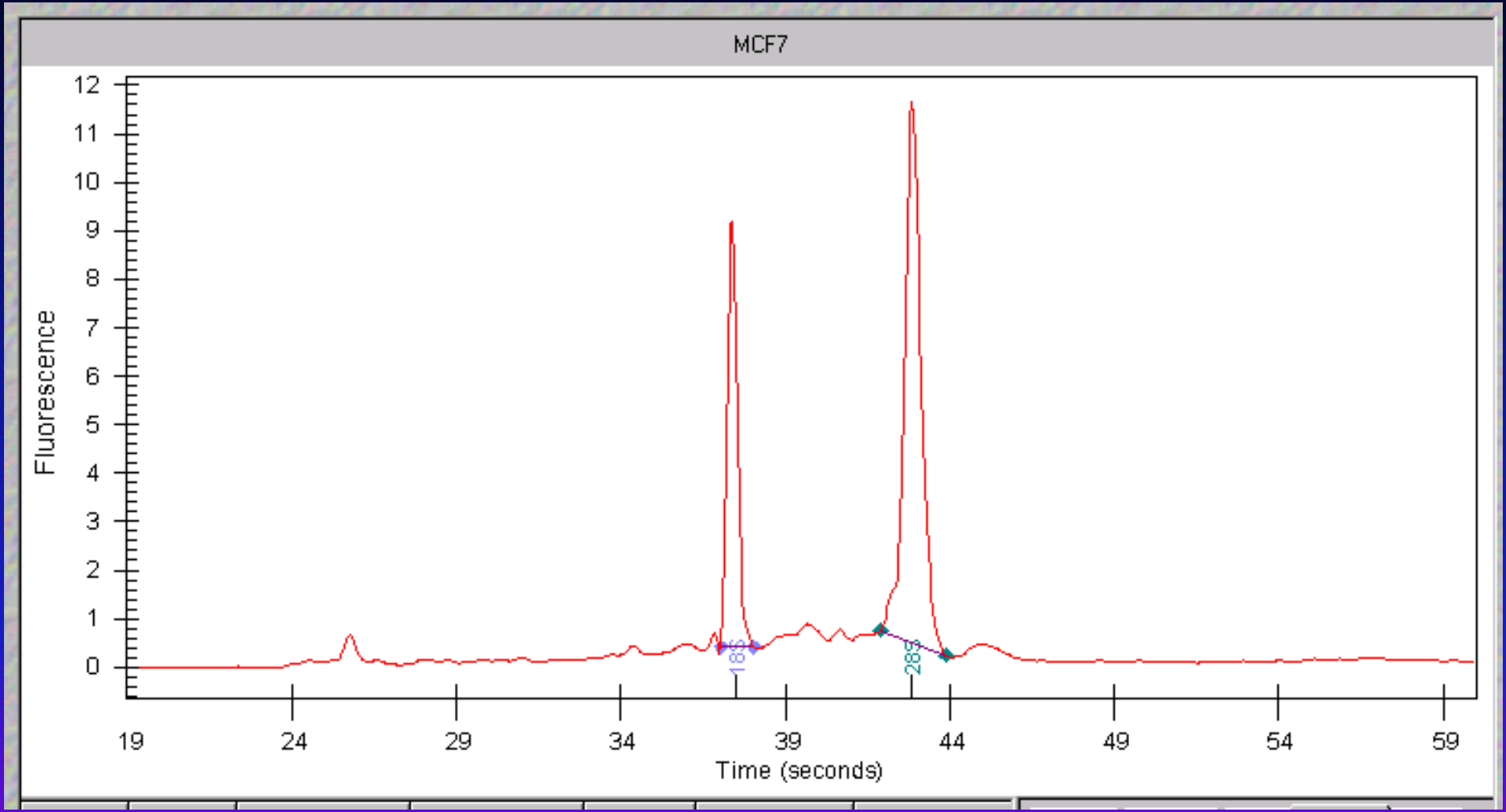
0, 0_2 005, 005_2 2.5, 2.5_2

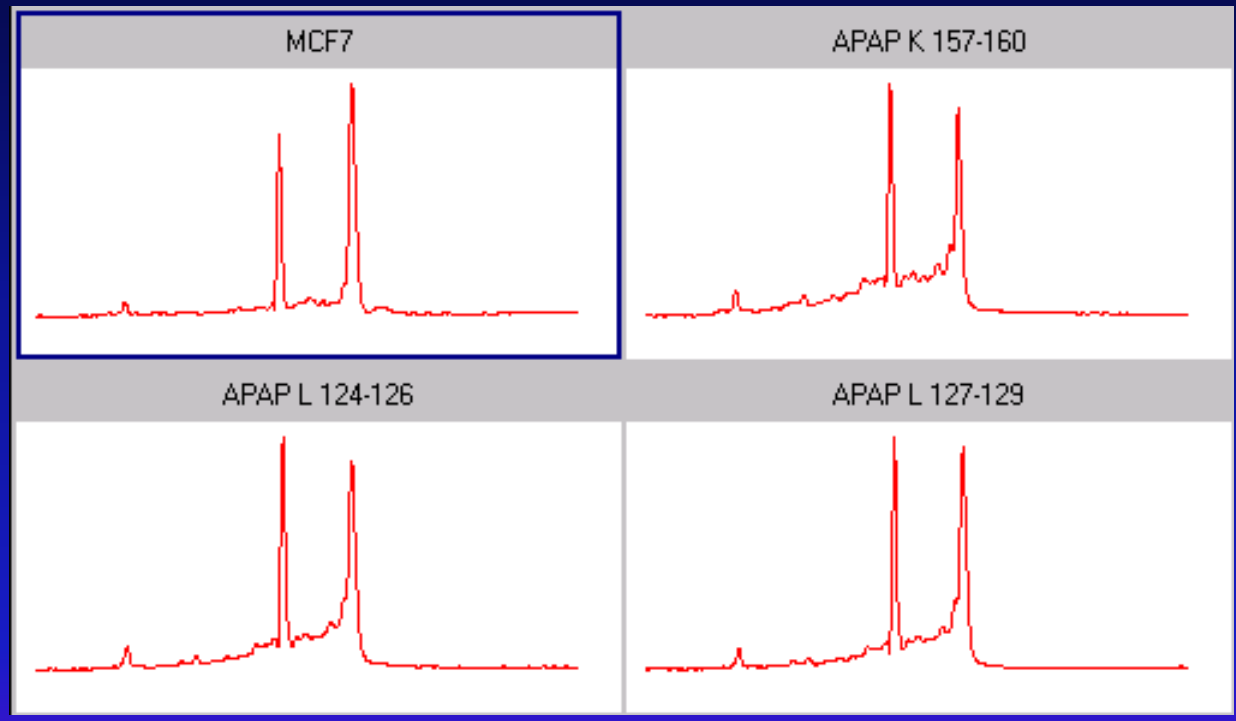
24 hr.

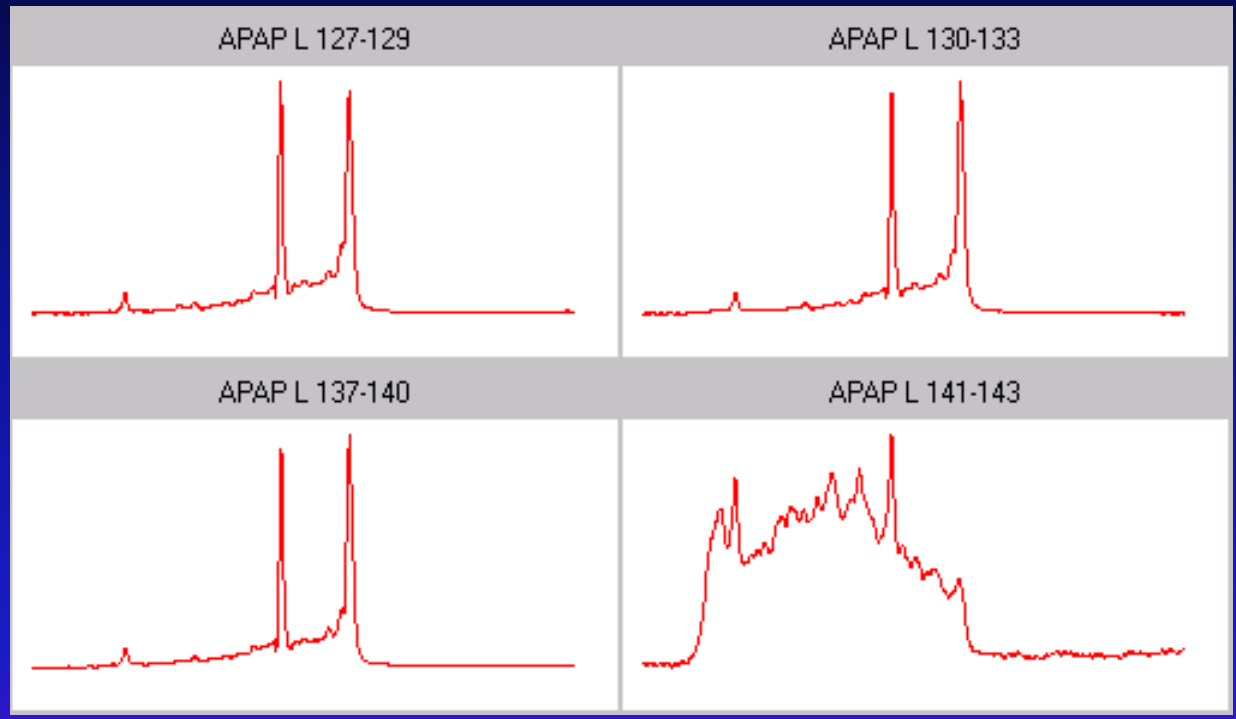
0, 0_2 005, 005_2 2.5, 2.5_2

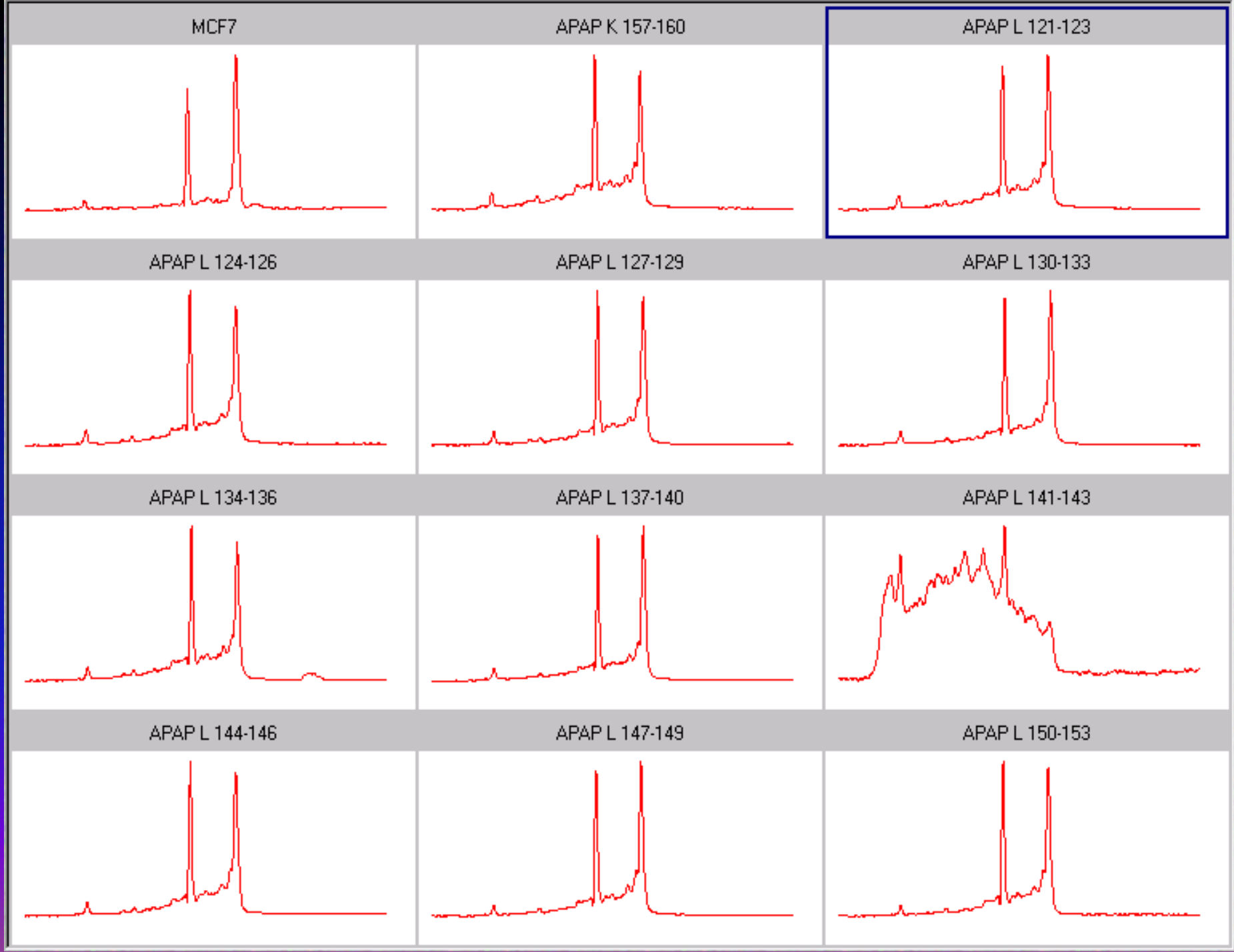
- RNA integrity
- diluted to 1 ug/ul
- 1 ug total per lane


Ladder
MCF7
APAP K 157-160
APAP L 121-123
APAP L 124-126
APAP L 127-129
APAP L 130-133
APAP L 134-136
APAP L 137-140
APAP L 141-143
APAP L 144-146
APAP L 147-149
APAP L 150-153
L
1
2
3
4
5
6
7
8
9
10
11
12


MCF7
Fluorescence
0
1
2
3
4
5
6
7
8
9
10
11
12
18S
28S
19
24
29
34
39
44
49
54
59
Time (seconds)


MCF7
APAP K 157-160
APAP L 124-126
APAP L 127-129


APAP L 127-129
APAP L 130-133
APAP L 137-140
APAP L 141-143


MCF7
APAP K 157-160
APAP L 121-123
APAP L 124-126
APAP L 127-129
APAP L 130-133
APAP L 134-136
APAP L 137-140
APAP L 141-143
APAP L 144-146
APAP L 147-149
APAP L 150-153

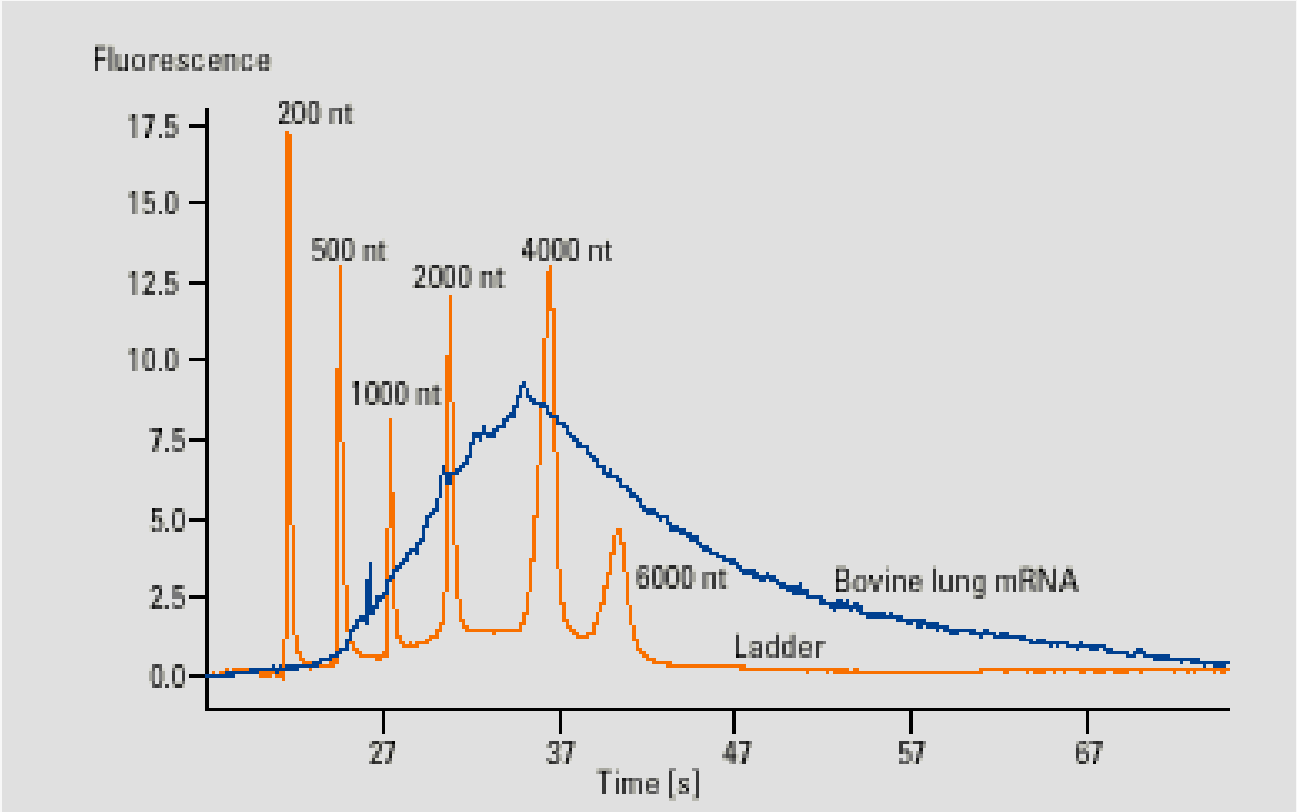
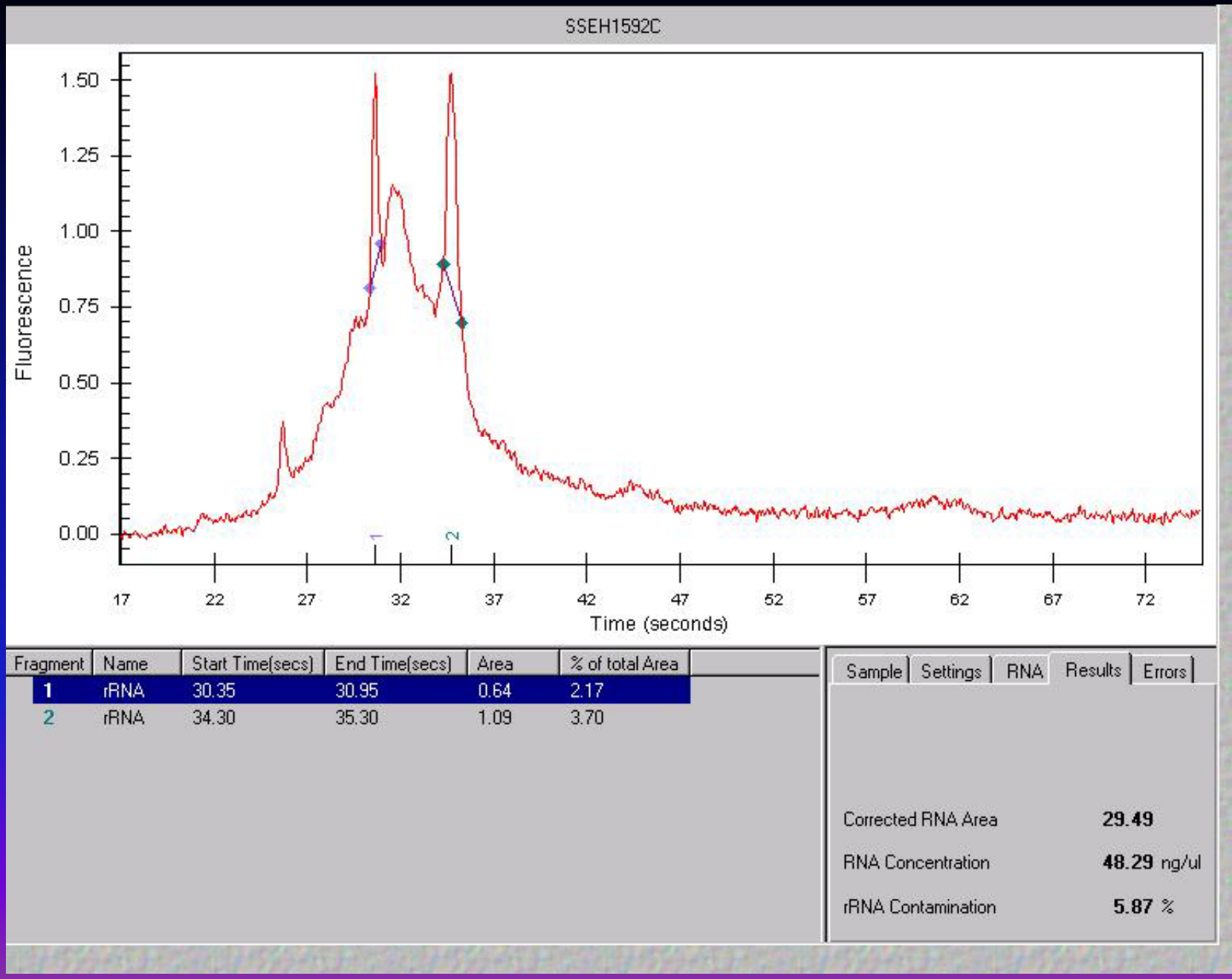


Figure 1

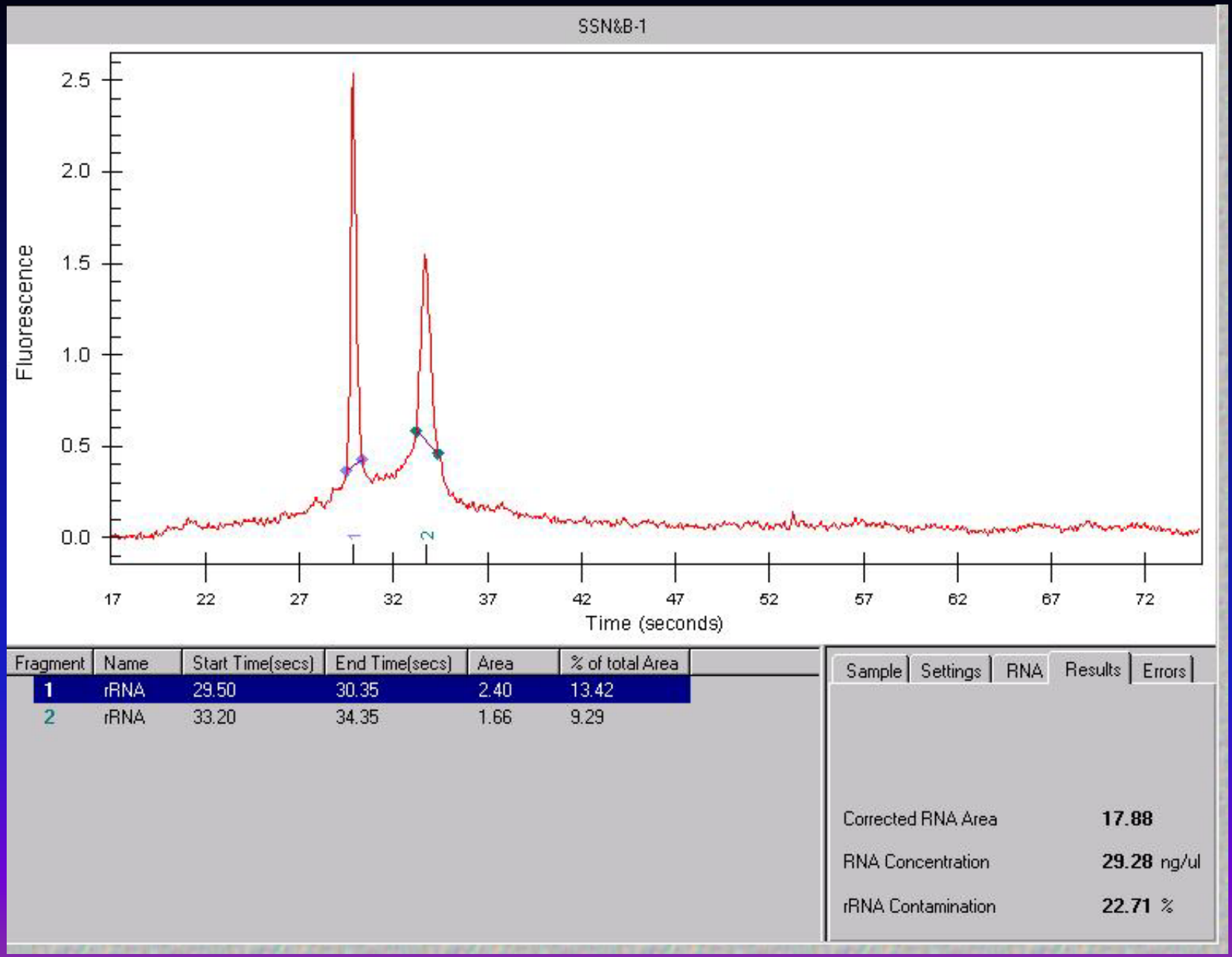
The relative amounts of transcripts falling in a certain size range can be estimated by overlaying the RNA 6000 ladder onto the electropherogram by holding down the control key while using the mouse to click on the ladder lane in the small gel-like image. The figure shows overlaid electropherograms of bovine lung mRNA, 250 ng/ µl, and the RNA 6000 ladder. The ladder contains six fragments of sizes 200, 400, 1000, 2000, 4000 and 6000 nucleotides. The relatively smooth character of the broad bovine lung mRNA peak is typical of a high quality mRNA sample free of ribosomal RNA contamination.



Fragment	Name	Start Time(secs)	End Time(secs)	Area	% of total Area
1	rRNA	30.35	30.95	0.64	2.17
2	rRNA	34.30	35.30	1.09	3.70

Sample | Settings | RNA | Results | Errors

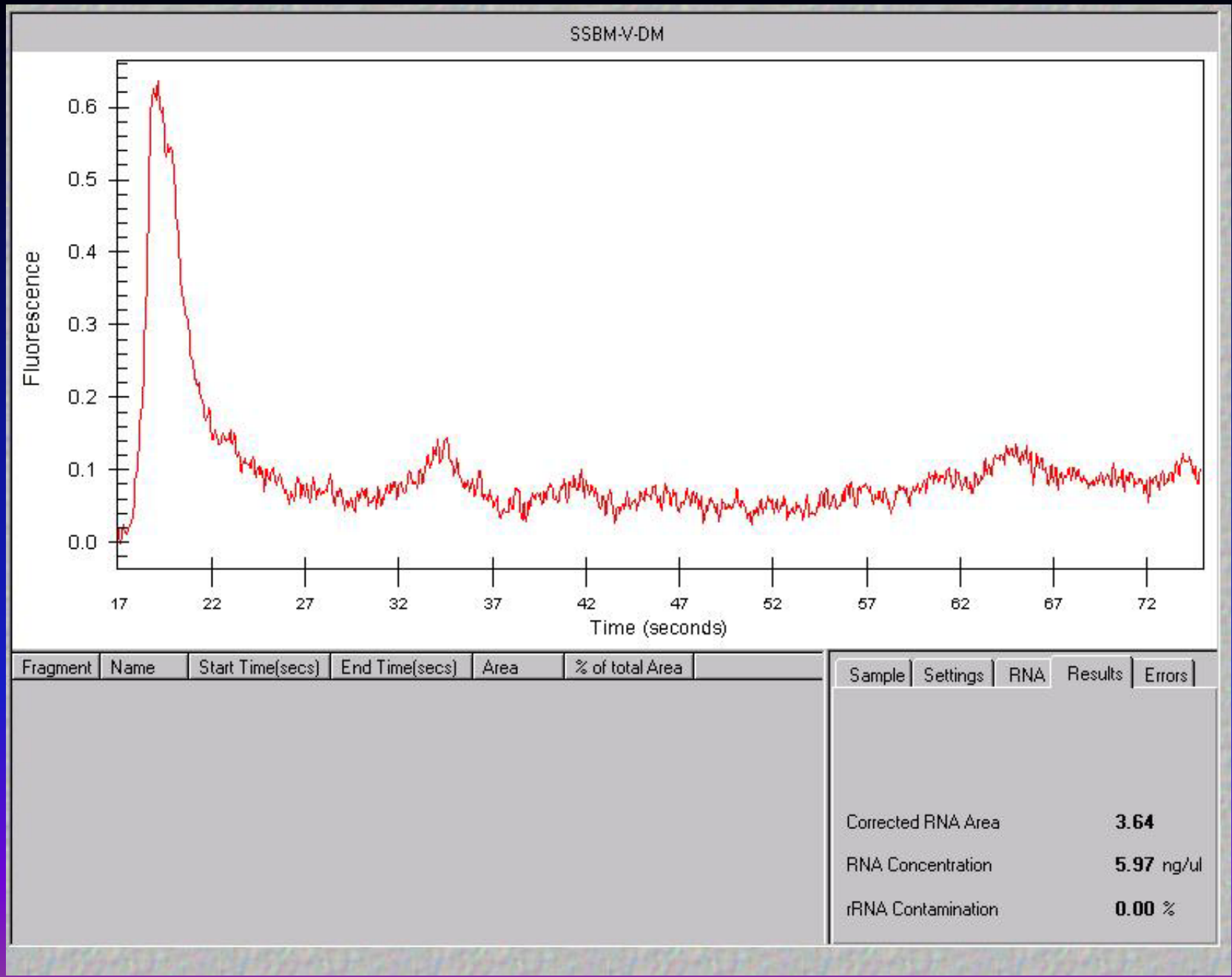
Corrected RNA Area	**29.49**
RNA Concentration	**48.29** ng/ul
rRNA Contamination	**5.87** %

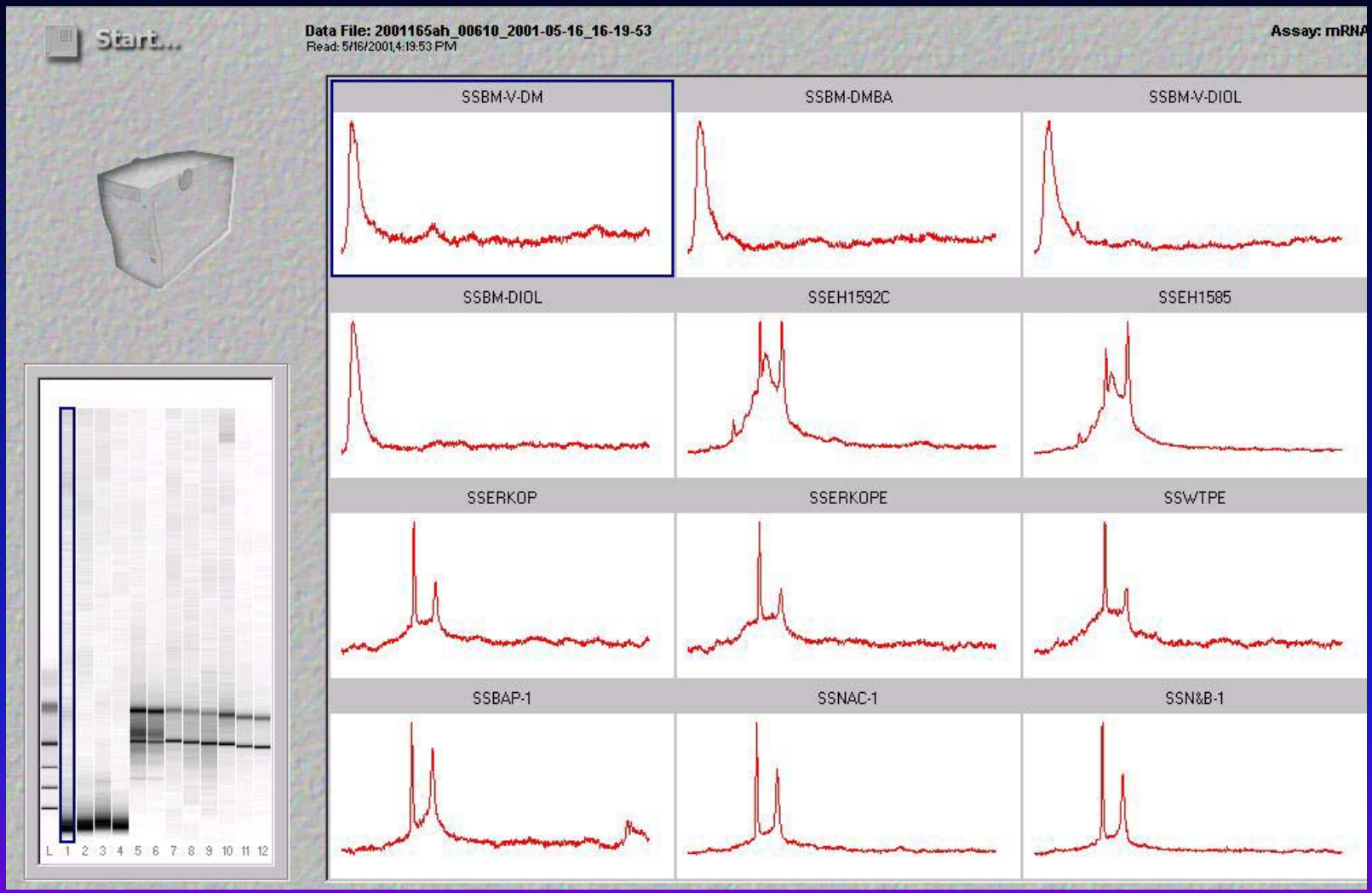


Fragment	Name	Start Time(secs)	End Time(secs)	Area	% of total Area
1	rRNA	29.50	30.35	2.40	13.42
2	rRNA	33.20	34.35	1.66	9.29

Sample | Settings | RNA | Results | Errors

Corrected RNA Area	**17.88**
RNA Concentration	**29.28** ng/ul
rRNA Contamination	**22.71** %


SSBM-V-DM
Fluorescence
0.6
0.5
0.4
0.3
0.2
0.1
0.0
17
22
27
32
37
42
47
52
57
62
67
72
Time (seconds)
Fragment
Name
Start Time(secs)
End Time(secs)
Area
% of total Area
Sample
Settings
RNA
Results
Errors
Corrected RNA Area
3.64
RNA Concentration
5.97 ng/ul
rRNA Contamination
0.00 %


Start...
Data File: 2001165ah_00610_2001-05-16_16-19-53
Read: 5/16/2001,4:19:53 PM
Assay: mRNA
SSBM-V-DM
SSBM-DMBA
SSBM-V-DIOL
SSBM-DIOL
SSEH1592C
SSEH1585
SSERKOP
SSERKOPE
SSWTPE
SSBAP-1
SSNAC-1
SSN&B-1
L 1 2 3 4 5 6 7 8 9 10 11 12

How should I submit samples?

- First, consider a pilot RNA prep
- Follow the instructions on the web page:
  - Gel and quantitation
  - Label tubes and sheet the same way
  - Notify us in advance of submission
  - Deliver samples on dry ice to a person
  - Label form neatly

http://dir.niehs.nih.gov/microarray

Protocols here- check date
Sample submission form
Previous user's group meetings

Technical Staff:

Jeff Tucker- bioengineer
Astrid Haugen- validation/special tox projects
Sherry Grissom-human, yeast, mouse basic studies
Danica Ducharme-robotics/ printing
Stella Sieber
Neysa Garner/Tomo Oshimura-STEPS

Jennifer Collins-web page

Acknowledge and Thanks to Julie Foley